The University of Chicago

QUANTITATIVE STUDIES ON NERVE REGENERATION IN AMPHIBIA

I. FACTORS CONTROLLING NERVE REGENERATION IN ADULT LIMBS

II. FACTORS CONTROLLING NERVE REGENERATION IN REGENERATING LIMBS

PART OF A DISSERTATION SUBMITTED TO THE FACULTY OF THE DIVISION OF THE BIOLOGICAL SCIENCES IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF ZOOLOGY

1937

By

RAYMOND LITWILLER

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QUANTITATIVE STUDIES ON NERVE REGENERATION IN AMPHIBIA [1]

I. FACTORS CONTROLLING NERVE REGENERATION IN ADULT LIMBS

RAYMOND LITWILLER [2]
Department of Zoology, The University of Chicago

TWO FIGURES

(Accepted for publication March 28, 1938)

Regeneration of nerves after transection is the result of a process of growth beginning from the cut ends of the proximal stumps left in connection with their cell bodies. Extensive bifurcation of the sprouts provides for an adequate supply of peripheral terminations. The mechanisms at play, however, are not understood.

Weiss ('31) reported a study of the innervation of two supernumerary fore limbs of an adult frog. He found that the number of nerve fibers connected with the normal and the two near-by supernumerary limbs was about three times as large distally as proximally. These results would indicate that an adjustment of the nerve number to satisfy an increased peripheral load can be accomplished by repeated peripheral division of axons. This for the first time suggests that the non-nervous periphery may affect the intensity of peripheral branching of outgrowing fibers in such a way as to produce a constant ratio between the size of the innervated periphery and the number of terminal nerve branches. Again,

[1] Presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Zoology, The University of Chicago.

The author wishes to express his appreciation to Dr. Paul Weiss for suggesting this work and giving much helpful advice and criticism throughout its progress.

[2] This work has been supported by a Biological Research grant to The University of Chicago from the Rockefeller Foundation.

Weiss ('37), when transplanting in axolotls a supernumerary limb next to a normal limb and assigning to the transplant, one of the three plexus nerves, found that the innervating fibers, irrespective of their original number, sent into the transplant a number of branches closely corresponding to the number of fibers in the near-by control limb. In these experiments, the transplanted limb seemed to have regulated the admission of new fiber branches within it, although the limb was differentiated and of normal size.

The work presented here is an attempt to analyze further the problem suggested by the findings of Weiss ('31, '37). For a preliminary account see Weiss and Litwiller ('37).

MATERIAL AND METHODS

The animals used were adult Japanese newts, Triturus pyrrhogaster (Boie). The method of attack was simply to transect the brachial nerves leading to the anterior limb and to modify in various ways, 1) the nerve supply to the limb, 2) the limb itself. The opposite limb of the same animal served as control.

The operations are illustrated in figure 1. Operation C presents the normal control; nerves were simply cut and allowed to regenerate back into the limb. In D the nerves were transected as in C, but in addition, their peripheral fragments were removed from the limb by pulling them out through the opening at the base of the limb, previously made when the nerves were transected. Inasmuch as these peripheral fragments after degeneration are supposed to be an excellent guiding substratum for regenerating fibers, a comparison between C and D was expected to yield information as to whether quantitative control of regeneration, if exhibited at all, was to be credited to these peripheral stumps, present in C, or to other limb tissues. In operations A and B, these same conditions of the periphery existed, but in addition an experimental reduction was made of the nerve supply available for regeneration. A reduction of roughly one-half was brought about by deviating the cut end of the fourth spinal

nerve into the shoulder muscle, m. dorso-humeralis, the tension of the muscle fibers holding the nerve in place. A comparison of A with C and of B with D was supposed to reveal whether the number of fibers available for innervation has any bearing on the number of branches actually penetrating into the limb.

After 120 days following the operations, the operated limbs together with their control limbs were removed, fixed in 10% formalin and treated by Bielschowsky's silver method for

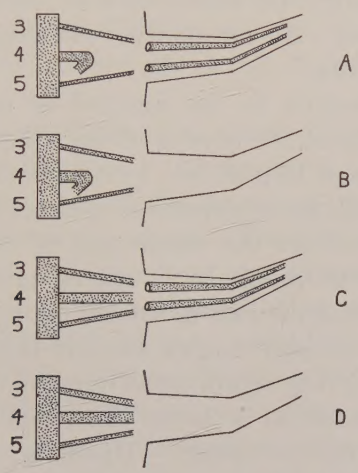

Fig. 1 Diagram to illustrate the four types of operations performed upon adult Triturus limbs.

quantitative studies at various levels of the limb. Three levels, at right angles to the axis of the limb, were selected. Level I was taken through the base of the limb, at the point where the proximal head of the humerus is clearly indicated; level II at the elbow, where the ulna first appears in view as more peripheral sections are studied; level III in the proximal wrist region where the three ossicles, radiale, intermedium and ulnare, first appear in the cross sections.

The nerve fibers were counted in the following manner: The number of fibers found in the main radial and ulnar trunks were obtained by making camera lucida drawings on

paper of each fiber seen in the bundle. Obviously this removes the likelihood of counting the same fiber twice. The scattered fibers were counted in the following manner: A 5-mm. glass micrometer scale was placed in the ocular of the microscope, so that it could be used as a stationary guide to delimit the strip of the microscopic field as the slide was moved from left to right by a mechanical stage. After the fibers found within this strip were counted, the field was moved so that an adjoining strip would be traversed and counted. Eventually the whole area of the limb cross section would be traversed. An automatic recording device was used to record the counts.

A possible objection to the method of counting scattered fibers is that fibers at the border of the strips may be counted twice. This was met in part by: 1) the fibers appearing under the end of the scale were never counted, since that same area would be traversed by the other end of the scale when an adjoining strip was studied; 2) scattered fibers form small groups often associated with mesenchyme cells and capillaries at various characteristic angles so that they would be easily recognized, should they again come into the field. Every tenth count was recounted at a later time, without knowing the magnitude of the earlier counts. The difference in percentage ranged from 1.5 to 11.9, averaging 5.9%.

The method to determine the areas of the cross sections was as follows: The outline of the section minus the skin was projected by means of a camera lucida on graph paper having units of 0.01 square inches. The number of units covered by the outline, divided by the square of the linear magnification of the camera lucida equals the actual size of the cross section. Since we are interested in comparative data only, the original arbitrary units will be used.

EXPERIMENTAL

The main point we wished to test was to see what effect the experimental reduction of the normal nerve number available for regeneration into the limb would have on the regenerative

repletion with fibers of limbs of constant size. The nerve source was allowed to vary, either alone or together with a change in the size of the limb.

At the same time, another problem could be investigated. If a quantitative limitation of nerve fiber regeneration into an adult limb should prove to be a fact, such influence could be ascribed to either the old degenerated nerve fiber fragments contained in the limb and serving as guiding paths, or to the total mass of limb tissues. In order to decide this question, the peripheral nerve fragments were experimentally removed from one group of limbs and the results thus obtained were compared with those limbs left in possession of their old nerve trunks.

Operations were performed on ninety Triturus, in combinations as shown in figure 1. Of the three levels which were studied, level I, the base of the humerus, was the most variable as far as area was concerned. The variability is probably due to the hazards of operation performed near this level and entailing the formation of scar tissue of varying extent. Another factor which might be a source of variability is the fact that level I was always chosen with reference to the greatest width of the humerus, regardless of the amount of trunk musculature found in the section. Therefore only levels II and III will be considered in regard to limb areas, while all three levels will be discussed in relation to the number of nerve fibers found at various levels of the limb.

The effect of proximal reduction of fiber supply on peripheral numbers

1. Regeneration from a full-sized proximal source. In table 1 the data on series TC are compiled. All limbs were functional at the time of their removal, 120 days after operation. Choosing case TC 2 as an example, we find at level I a count of 869 nerve fibers in the operated limb as compared with a count of 1133 fibers in the control limb, showing a deficit of 264 fibers, or 23.3%. Again, at level II the number of nerve fibers was found to be 587 in the operated limb as compared with 768 in the control limb, the difference being 181 or 23.5%.

TABLE 1

Operation TC

ANIMAL NO.	BODY LENGTH	FUNCTION	LEVEL I BASE OF LIMB			LEVEL II HUMERUS-ULNAR JUNCTION			LEVEL III WRIST		
			Operated limb	Per cent of difference	Control limb	Operated limb	Per cent of difference	Control limb	Operated limb	Per cent of difference	Control limb
Part (1), number of nerve fibers (N) (Bielschowsky silver)											
	mm.										
TC 2	60	+	869	—23.3	1133	587	—23.5	768	252	—27.4	347
TC 4	65	+	1359	—13.5	1572	564	—44.8	1021	145	—74.7	573
TC 6	54	+	1190	— 1.9	1214	812	— 7.2	875	234	+15.8	197
TC 7	55	+	848	—22.1	1089	523	—23.9	687	205	—28.8	288
TC 8	49	+	1324	+ 1.0	1312	601	—13.3	693	42	—89.7	407
TC 9	60	+	776	—27.8	1075	527	—37.7	847	258	—33.0	385
TC 10	70	+	1123	—11.9	1275	514	—33.0	769	225	—26.2	305
Averages			1069	—14.2	1238	589	—27.2	808	194	—37.7	357
Part (2), area of corresponding sections (A)											
TC 2	60	+	1157	—36.9	1833	1408	—24.3	1862	968	—39.3	1595
TC 4	65	+	1696	—22.3	2182	1917	—11.4	2164	1446	+ 4.6	1379
TC 6	54	+	1357	—17.2	1639	1420	— 2.7	1459	991	—16.2	1182
TC 7	55	+	1207	—11.7	1367	974	—17.8	1185	782	—10.3	872
TC 8	49	+	1300	—20.0	1627	934	—14.9	1097	1056	— 2.2	1080
TC 9	60	+	1185	— 7.7	1284	1201	— 2.4	1230	406	—62.7	1089
TC 10	70	+	1565	—17.1	1889	1504	—19.2	1861	288	—80.7	1489
Averages			1352	—19.0	1688	1336	—13.2	1551	848	—30.0	1240

At level III, 252 nerve fibers in the operated limb compared to 347 in the control limb, a difference of 95 fibers, or 27.4%.

However, the cross sections at levels II and III are also smaller than the corresponding sections of the control limb (table 1). In case TC 2, at level II we find a reduction in the area of the operated limb, compared to the control, of 24.3% which by accident is very close to the amount of nerve fiber deficit of 23.5%. At level III, the respective values are 39.3% and 27.4%. The average reduction in cross sectional area of the two distal levels II and III in the operated limbs of all seven tabulated cases is 13.2% and 30.0%. Since the growth of these adult animals within the period of observation, 4 months, is negligible, the conspicuous size difference is evidently the expression of actual atrophy in the denervated limb rather than of retarded growth. Tower ('35), has presented data to show that denervation entails atrophy of mammalian skeletal muscle. Our data have revealed a deficit in the operated limb in regard to both size of limb, and content in regenerated nerve fibers.

A comparison of the averages of all the TC cases brings out the following points: 1) At all three levels, there is a deficit in the number of nerve fibers found as compared to the controls, namely, (I) 14.2%, (II) 27.2%, and (III) 37.7%. 2) At levels II and III there are also corresponding reductions in area as compared to control sections.

Figure 2 represents case TC 7 as an example of operation C, in graphic form; while there is a significant deficit in the number of nerve fibers from A′ to B′, there is also a decrease of the areas of the respective cross sections.

Our data are not adequate to decide the question whether or not there is an immediate relation between the atrophy of the limb and the deficit in the number of nerve fibers that have regenerated into it. One must consider the possibility that the time allowed for the experiments may not have been sufficient to permit the process of nerve regeneration to run its full course; after another month larger nerve fiber numbers might have been counted. This is suggested by the greater

deficit in fibers at level III in the TC averages. It should be recalled, however, that in the studies by Weiss ('37), of the innervation of transplanted limbs, a similar proportionality between the actual size of the transplant and its content of regenerated nerve fibers has been recorded after periods of time had elapsed which by far exceeded the time required for complete re-innervation.

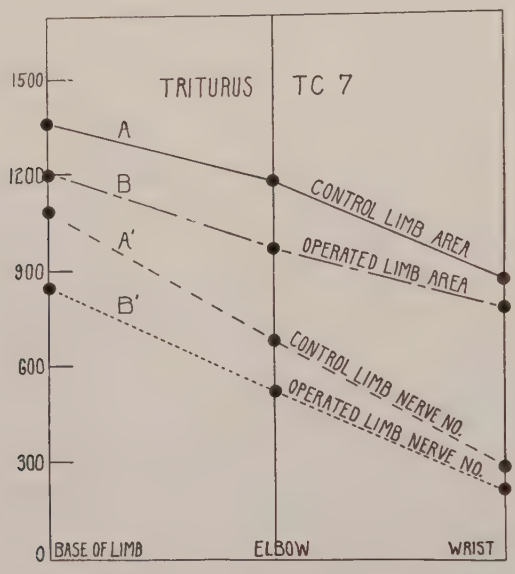

Fig. 2 A sample case of operation C.

Whatever the answer may be in our cases, the data furnish a standard with which to compare the amount of fiber regeneration obtained after elimination of either proximal or peripheral nerve trunks or both.

2. Regeneration from a reduced proximal source. In a comparison of series TA with TC (tables 1 and 2) it will be observed that the deficit in the number of nerves on the operated side in the TA series was 20.5% at level I, only slightly more than the deficit of 14.2% for the TC series. However, at the more distal levels II and III, more marked deficits occur. At level II the TA series averages a 50.1% deficit in

TABLE 2

Operation TA

ANIMAL NO.	BODY LENGTH	FUNCTION	LEVEL I BASE OF LIMB			LEVEL II HUMERUS-ULNAR JUNCTION			LEVEL III WRIST		
			Operated limb	Per cent of difference	Control limb	limb Operated	Per cent of difference	Control limb	Operated limb	Per cent of difference	Control limb
Part (1), number of nerve fibers (N) (Bielschowsky silver)											
	mm.										
TA 1	56	+	833	—34.3	1268	224	—76.7	969	152	—22.9	197
TA 2	58	+	997	—12.2	1136	385	—52.2	806	270	—41.7	463
TA 6	60	+	1327	—12.4	1514	483	—42.7	843	165	—64.4	463
TA 7	58	+	1384	+ 5.3	1311	391	—48.1	753	174	—58.6	421
TA 15	57	+	656	—48.9	1285	591	—30.4	849	214	—56.9	496
Averages			1039	—20.5	1302	415	—50.1	844	195	—49.0	408
Part (2), area of corresponding sections (A)											
TA 1	56	+	1147	—45.5	2103	1240	—16.5	1485	982	—25.9	1323
TA 2	58	+	1161	—15.0	1366	1134	— 0.4	1138	783	—20.0	979
TA 6	60	+	2017	+28.1	1450	902	—25.8	1215	888	—17.2	1072
TA 7	58	+	1517	— 1.8	1545	1046	—20.1	1309	967	+ 0.4	963
TA 15	57	+	1610	+ 9.8	1452	1345	— 3.0	1387	1050	—10.1	1168
Averages			1490	— 5.0	1583	1133	—13.2	1306	934	—14.5	1101

the number of nerve fibers compared to the average for TC of 27.2%. Again, at level III the percentages are 49.0 for TA to 37.7 for TC.

There is a reduction in the area of the cross sections of TA which averages 13.2% and 14.5% for levels II and III respectively, which approaches closely the average reductions in area of the level II for TC namely, 13.2%, but is less than the percentage of 30.0 for level III of TC. In view of the small number of cases these size differences between TA and TC cannot be considered as significant.

With the deficit in the number of nerves in operation C (nerves merely transected) serving as control, we may compare TA averages directly with TC; differences in results can then be attributed to the reduction of potential nerve supply in operation A due to the deviation of the fourth nerve. If tables 1 and 2 are analyzed from this point of view, it will be found that in the TA series there is a relatively greater deficit in the number of nerve fibers at the two distal levels. However, the difference is least at the most proximal level where we count most nearly the actual number of regenerated fiber branches. The fact that the difference between TC and TA at level I amounts to only 6.3% seems to indicate that the number of nerve fiber branches regenerating into the limbs is not determined by the number of proximal fiber stumps from which the branches arise, since the elimination in series TA of about half the fiber source available in series TC has failed to produce a corresponding peripheral reduction. On the other hand, as indicated by the greater deficit in the more distal levels, the regenerating fibers have not grown out as far on an average in TA as they have in TC. Whether this difference might have been smoothed out, if more time had been allowed to the experiments, cannot be decided here.

The effect of removal of peripheral fiber tracts from the limb upon peripheral numbers

1. Regeneration from a complete proximal source. In operation D, after transection of the nerves as in C, the peripheral fragments were removed from the limbs. A comparison between C and D, therefore, may yield information concerning the possible role of peripheral sheaths in the quantitative control of fiber outgrowth. It will be observed in table 3, that at every level there is a greater deficit in the number of nerve fibers of the operated limbs of the TD cases than the average deficit of the corresponding levels in the TC series (table 1). For example, at level I, at the base of the limb, the deficit in the number of nerve fibers averages 38.6% in the TD series compared with an average deficit of 14.2% in TC, a difference of 24.4%. Again at level II, the TD series show a deficit of 69.0% in the number of nerve fibers compared to an average deficit of only 27.2% in the TC group, a difference of 41.8%. Level III of TD shows an average of 72.4% deficit as compared with the deficit of 37.7% for the TC cases, or a difference of 34.7%.

If a similar comparison of the two series is made with regard to the area of the cross sections, we find that the averages at level II of the TD (table 3) show a reduction of only 7.7% compared to the reduction of 13.2% for the same level of the TC series. At level III there is a somewhat greater differential between the averages for TD, 14.9% and for TC, 30.0%. Both the TD and TC series furnish results which agree in that a reduction in cross sectional area does occur after operation, but there is no differential reduction, due to the removal of the nerve fragments from the limb. It is possible that the operative removal of peripheral nerve fragments from the limb in TD caused a slight degree of tissue regeneration. This is not likely since in TB, similar to TD in regard to removal of peripheral fragments, the opposite resulted, namely a decrease in cross sectional areas when compared to TC. These differences may be due to random fluctuations.

TABLE 3

Operation TD

ANIMAL NO.	BODY LENGTH	FUNCTION	LEVEL I BASE OF LIMB			LEVEL II HUMERUS-ULNAR JUNCTION			LEVEL III WRIST		
			Operated limb	Per cent of difference	Control limb	Operated limb	Per cent of difference	Control limb	Operated limb	Per cent of difference	Control limb
Part (1), number of nerve fibers (N) (Bielschowsky silver)											
	mm.										
TD 1	67	+	579	—59.0	1412	463	—49.8	923	415	—21.7	530
TD 3	67	+	924	—23.8	1213	347	—42.6	605	156	—51.4	321
TD 4	62	—	788	—14.3	920	36	—92.7	495	30	—83.5	182
TD 5 [1]	68	—	530	—49.8	1056	86	—84.5	555	14	—95.8	332
TD 6 [1]	53	+	917	—29.9	1309	296	—55.8	669	58	—86.5	429
TD 7	54	—	506	—54.0	1100	73	—88.3	624	18	—95.7	420
Averages			707	—38.6	1182	216	—69.0	645	115	—72.4	369
Part (2), area of corresponding cross sections (A)											
TD 1	67	+	1231	—31.1	1787	1413	+ 0.6	1405	1219	— 0.08	1220
TD 3	67	+	2092	+17.9	1718	1715	+11.7	1514	1381	+ 5.9	1300
TD 4	62	—	2643	+15.7	2228	1294	—19.6	1610	1173	—14.8	1376
TD 5	68	—	1628	+ 2.9	1580	1205	—10.9	1353	903	—26.8	1234
TD 6	53	+	1566	+ 4.5	1495	1113	— 3.2	1150	826	—25.2	1105
TD 7	54	—	2227	+22.6	1723	1013	—24.9	1349	834	—28.6	1168
Averages			1897	+ 5.4	1755	1258	— 7.7	1396	1056	—14.9	1233

[1] Limb lacked fingers.

A comparison of tables summarizing cases TC and TD (tables 1 and 3), will reveal that while none of the TC operations resulted in limbs having impaired movements or anatomical deformities as lack of fingers, some of the TD series showed defects of this nature. It must be remembered that all operated limbs possessed originally normally formed fingers, so that in those cases where fingers were not found, a loss of the structure is indicated rather than the lack of its formation. In those TD cases showing the greatest deficit, as for example TD 5 (table 3), of 84.5%, with only a 10.9% reduction in area, there was also a lack of function and fingers were absent. TD 4 and TD 7 both were non-functional and also showed marked deficits, particularly at levels II and III. Weiss ('25), clearly demonstrated in urodele Amphibia that the nervous system at the base of the limb was necessary for limb regeneration. It is very probable that in our TD series the failure in some cases to regenerate fingers can be attributed to a gross deficit in limb innervation in the periphery.

2. Regeneration from a reduced proximal source. In series TA, the effect of the reduction of the proximal nerve source was studied. Series TD revealed the effects of the removal of the distal nerve trunks. In series TB, both these interferences were combined, that is, the proximal nerve stumps, minus the fourth nerve, were allowed to regenerate into a limb from which the old nerve fragments had been removed.

Under these conditions, a deficit was found in the number of nerve branches in the operated side amounting, at the three levels, to 41.1%, 67.6% and 72.5%, respectively (table 4). This compares with 38.6%, 69.0% and 72.4% in series TD, where the full proximal nerve supply was available. It can be seen, therefore, that the reduction of the proximal nerve source in series TD has had no depressing effect on the number of nerve branches regenerating into the limb. This same conclusion has been reached above from a comparison of series TA and TC. The number of nerve branches entering the limb is not determined simply by the number of proximal fibers from which they originate but seems to be controlled by peripheral factors.

TABLE 4

Operations TB

ANIMAL NO.	BODY LENGTH	FUNCTION	LEVEL I BASE OF LIMB			LEVEL II HUMERUS-ULNAR JUNCTION			LEVEL III WRIST		
			Operated limb	Per cent of difference	Control limb	Operated limb	Per cent of difference	Control limb	Operated limb	Per cent of difference	Control limb
Part (1), number of nerve fibers (N) (Bielschowsky silver)											
	mm.										
TB 1	61	+	556	—51.8	1153	259	—67.3	793	220	—53.5	474
TB 4	61	+	967	—23.6	1266	394	—51.3	809	120	—74.0	462
TB 6 [1]	54	—	613	—57.1	1428	406	—46.9	763	128	—59.8	318
TB 9 [2]	59	—	346	—68.7	1107	153	—78.2	701	37	—85.5	255
TB 11 [3]	60	—	966	— 4.5	1012	45	—94.1	769	8	—89.5	76
Averages			690	—41.1	1193	251	—67.6	767	103	—72.5	317
Part (2), area of corresponding sections (A)											
TB 1	61	+	1650	+ 7.4	1528	1135	— 5.6	1203	845	—19.8	1053
TB 4	61	+	2695	+10.4	2414	202	—86.5	1499	113	—90.7	1206
TB 6	54	—	1755	+27.3	1275	1073	—12.1	1221	1078	+ 0.9	1068
TB 9	59	—	1279	—13.5	1478	1165	— 2.8	1199	761	—10.0	846
TB 11	60	—	3124	—21.5	2452	1149	— 8.5	1255	1036	+24.4	783
Averages			2100	+10.6	1829	944	—23.1	1275	766	—19.0	991

[1] Wrist not functioning. Elbow movement jerky.

[2] Wrist not functioning. Movements jerky.

[3] Movement at shoulder only.

On the other hand, it will be remembered that the drop in the number of nerve fibers from more proximal to more distal levels was much greater in series TA than in series TC, indicating a failure of the regenerating fibers in TA to reach, on an average, as distant points as they would have reached in undisturbed regeneration. The possibility that this difference might have disappeared, if more time had been allowed for the experiments, was mentioned above. It should not obscure, however, the fact that the number of regenerating fibers counted at the entrance of the limb, level I, seems to be independent of the size of the source within the limits examined.

From a comparison of tables 2 and 4 (TA and TB), it will be seen that the nerve deficit is much greater in the TB experiments, where the nerve fragments within the operated limb had been removed, than in the TA series, the percentage being, at level I, 20.5 and 41.1 respectively. At level II the difference is less marked, the TA average being 50.1% against 67.6% in TB, or 17.5% less. At level III, TA averages 49.0% while TB shows 72.5%, a difference of 23.5%.

At the same time, there is no significant difference between the two series in regard to the area of the cross sections at levels II and III. At level II, TA averages 13.2%, compared to a difference of 23.1% for TB. Again at level III, TA is 14.5% to 19.0% for TB.

Again, as in series TD, a number of non-functional and in some cases, malformed limbs were found in the TB series, in which nerve trunks were removed from the limb. For example, table 4, case TB 11, the distal part of the operated limb became necrotic, then regenerated but lacked fingers. The only movements of the limb were movements of the whole limb, the flexion and extension of the elbow being absent. Of the five cases reported of TB, only TB 1 and TB 4 were completely functional at the time of removal—120 days. This is in contrast to the TA series (peripheral nerve stumps allowed to remain), where all of the operated limbs had recovered function by the time of removal for study. There seems to be some correlation between extremely low numbers of nerve

fiber branches and malformed limbs lacking function. This is probably due to both atrophy and lack of nerve activity required for regeneration of fingers.

A comparison of the averages of all four series, TA, TB, TC and TD in table 5, shows that at every level there is a greater deficit in the number of nerve fibers found within the limb, when the peripheral nerve tracts have been removed from the limb. For example, in the TC series, nerves being merely transected, with a full proximal supply present, an average deficit of 14.2% occurs in the operated limb at level I,

TABLE 5

A comparison of the averages of TA, TB, TC and TD series

	PERIPHERAL NERVE TRUNKS NOT REMOVED FROM THE LIMB				PERIPHERAL NERVE TRUNKS REMOVED FROM THE LIMB			
	Level of count	Operated nerve number	Control nerve number	Difference	Operated nerve number	Control nerve number	Difference	
				%			%	
Proximal nerve supply complete TC	(I) Base of limb	1069	1238	14.2	707	1182	38.6	
	(II) Elbow	589	808	27.2	216	645	69.0	TD
	(III) Upper wrist	194	357	37.7	115	369	72.4	
Proximal nerve supply reduced TA	(I)	1039	1302	20.5	690	1193	41.1	
	(II)	415	844	50.1	251	767	67.6	TB
	(III)	195	408	49.0	103	317	72.5	

while the same level in the TD series, where in addition, the peripheral trunks were removed, the average deficit is 38.6%. Similar trends characterize levels II and III (see table 5). Clearly then there is a greater deficit in the averages of the TD group in which the peripheral trunks have been removed.

The TA and TB groups are in the same mutual relation as TC and TD, the difference lying in the presence or absence inside the limb of peripheral nerve trunks. However, the TA-TB series differs from the TC-TD group in that the size of the proximal nerve source has been reduced in the former.

At each level, the TB average deficit is larger than the deficit of the same level in the TA group. Obviously, the removal of the distal stumps of the sectioned nerves has a much more marked depressing effect on the regenerated fiber number than a reduction in number of proximal stumps available for regeneration.

DISCUSSION

Operation C has served as the standard for comparing operations A, B and D. Mere transection of the nerves proximal to the brachial plexus yields, after 120 days, a deficit of 14.2% (I), 27.2% (II), and 37.7% (III), in the number of nerve fiber branches found at the three levels of the operated limb when compared to the control side (table 1). However, there is also a significant reduction in the size of the operated limb as indicated by the reduction in the area of corresponding cross sections at levels II and III (13.2% and 30.0%, respectively).

The reason for this deficit in fiber numbers and reduction in size of limb in the TC series is still unknown. It may be that the size of the limb at the time the fiber branches are ready to enter the limb (reduced, due to atrophy) may be a factor in interpreting the results of operation C. The atrophy of the operated limb might be explained by the fact that for weeks after transecting the nerves, the limb lacks both motor and sensory innervation. The atrophy of muscles then would result in a general reduction in cross sectional areas as determined by our methods. Tower ('35), working with mammals (cat) has shown that muscles which have been denervated, show a definite reduction in the average diameter of individual muscle fibers, but that the total number of muscle fibers remain as large as before denervation. These results in mammals definitely indicate the role played by the nervous system in muscle maintenance. Weiss ('25) clearly indicated this role of the nervous system in work with urodele limb regeneration. The data presented in this paper in connection with operation C fall in line with their results.

Operation C, then, is essentially a control, the results of which can be used in analyzing the TA-TD series. The results of operation A when compared to C (tables 1 and 2) indicate slightly greater deficits at the two distal levels but at the proximal level there appears no appreciable difference. This indicates that even with a reduction of about 50% in the proximal potential supply to the operated limbs in A, almost as large a number of fiber branches are found entering the limbs as in C with the full source intact. Weiss ('37) has obtained similar results with transplanted Amblystoma limbs. The deficit in the two more peripheral levels in TA when compared to TC might be explained by the fact that the nerve fiber branches have not had sufficient time to reach the periphery. Since the limbs contained the old nerve trunks, this seems unlikely. It may be that we are dealing here with the same phenomenon which was found in the TC series, but merely more marked, due possibly to general effects of a more serious operation, and involving a greater delay before the limb became functional.

Analysis of the results of the TD series when compared to the TC group, definitely show that a smaller number of nerve fiber branches have regenerated in an operated limb from which the nerve trunks had been removed, when compared to operated limbs whose nerve stumps have been allowed to remain during the 120 days. Again, after reduction of proximal supply in the TA and TB series, the series in which the peripheral trunks have also been removed shows the greater deficit in the number of nerve fiber branches at the three levels.

Our data simply answer the question of whether there was a smaller deficit of regenerating nerve fibers due to the presence of the degenerated nerves (Buengner's cords) at 120 days after operation. The question of whether the branches would have, at a later time, filled the limb to the same degree as was found in the TC series is still unsettled. In other words, perhaps the TD series represent regenerating nerve branches of a younger age and, therefore, the TD group would not be expected to fill the limb to the degree found in the TC

series. Cajal ('28), Forssman (1898 and '00), Dustin ('10), Boeke ('21), and others have presented data to show that degenerated nerve sheaths affect the rate of nerve regeneration. Williams ('30), working with amphibian larvae (sixty-one cases) finds that axons which did not enter deserted sheaths grew at a rate of 2.95 μ per hour; those which did enter the sheaths showed a rate of growth of 8.5 μ per hour —a substantial increase. Although direct comparison of regenerating nerves of larvae with regenerating nerves within limbs of adult operated animals must be made with caution, it seems probable that such a differential rate might explain the differences found between operations C and D as well as A and B.

An important point which is shown by a comparison of the TC series with TD and TA with TB, is the fact that even in the absence of the old nerve trunks, there is a control to a degree at least, of the number of nerve fiber branches which are allowed to enter the operated limb. For example, in the TD series, where the nerve trunks were removed from the limb, the average deficit at level I was only 38.6%, or stated in another way, where the control limb before operation contained on the average 1182 fibers, the operated limb with the nerve trunk removed, still allowed 707 fibers to again enter, or about three-fifths of the normal number. The picture of the TB series is essentially the same.

The strongest indication that the agents controlling the nerve fiber invasion are present within the limb is the fact that at no time was there found within the limb a mass production of nerve fibers greatly above the normal number. If the formation of new fibers within the operated limb were entirely a random branching, an excess might be expected to occur. Although increased numbers were found proximal to the base of the limb, only normal or sub-normal numbers occurred within the operated limbs, regardless of the type of operation.

SUMMARY

1. In Triturus, limb nerves were transected and allowed to regenerate, either without further interference—operation C —or, in addition, removing the peripheral nerve trunk—operation D—or reducing nerve fiber supply to the limb—operation A—or in addition, removing the peripheral nerve trunks from the limbs—operation B.

2. Eliminating the fourth spinal nerve, the largest of the plexus, as a source of nerve fiber regeneration by deviating it into the shoulder muscles has not resulted in a corresponding deficit of regenerated nerve fiber branches within the operated limb. Therefore, the number of fiber branches regenerating into a limb is not primarily determined by the number of nerve fibers contained in the proximal trunks available for regeneration.

3. In spite of the removal of the nerve trunks from the operated limb, the number of nerve fibers admitted into the operated limb is about three-fifths of the number admitted into the control limbs, so that there is considerable control of nerve fiber ingrowth by the limb, even in the absence of the old nerve sheaths.

4. In none of the operations was the average number of regenerating fibers admitted into the limb above the normal average found at those levels. If nerve regeneration into the limb were a random mass regeneration, we would expect to find more than the control number.

5. The results of all these experiments demonstrate the existence within the limb tissues of factors controlling the number of nerve branches admitted to their terminal fields. Owing to the activity of these factors, the number of regenerated nerve fibers within a limb approximates to a certain degree the original number, regardless of the size of the nerve source, point 2. The controlling factors cannot be assumed to reside solely in the old nerve sheaths, because the removal of the latter, point 3, although impairing (delaying) nerve regeneration, does not abolish the tendency to establish normal numerical relations.

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QUANTITATIVE STUDIES ON NERVE REGENERATION IN AMPHIBIA [1]

II. FACTORS CONTROLLING NERVE REGENERATION IN REGENERATING LIMBS

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EIGHT FIGURES

The quantity of peripheral innervation is the product of two factors, one being the number of central nerve cells issuing peripheral fibers and the other being the amount of peripheral branching of the fibers. Studies to examine the former factor, the quantitative development of the nerve centers, have been made by a number of investigators, largely by modifying the amount of tissue to be innervated during embryonic stages. Detwiler ('20, '30), Schwind ('31), Carpenter ('32), have clearly shown that removal or addition of a limb affects the number of spinal ganglion cells formed during embryonic development. Shorey ('09) and Hamburger ('34), found that in higher vertebrates spinal cord development also depends to a certain degree on the size of the periphery.

As regards the branching of peripheral nerve fibers in response to varying loads of tissue, Weiss ('31), reported on a study of the innervation of two supernumerary fore limbs of an adult frog. He found that the number of nerve fibers connected with the normal and two near-by supernumerary limbs was about three times as large distally as

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proximally. This suggests that an adjustment of the motor nerve number to satisfy an increased peripheral load can take place by repeated axonal divisions. The writer ('38), has found that there is a definite control exerted by Triturus limbs upon the number of regenerating nerve fiber branches admitted rather than a random mass invasion of fibers into the limb.

A more direct attack of the problem of causal factors concerned in peripheral nerve branching can be made by the study of regenerating limbs of adult urodeles. The process of regeneration is in many respects similar to limb development during embryogenesis, but there are also some striking differences (Weiss, '30). Aside from the epithelial wound covering, nerve fibers, possibly blood vessels, and some muscles, all the tissues of the regenerate differentiate in situ from a mesenchymal blastema of local origin. The outstanding differences between the ontogeny and regeneration can be summarized as follows: 1) There is an enormous number of cells contained in the regeneration blastema as compared to the number forming the limb bud during embryogenesis. 2) There is a higher proportion of intercellular materials during regeneration. 3) Vascularization is present during regeneration enabling the circulation of hormones and other substances which are probably not present during early embryogenesis. 4) The nervous system is indispensable to regeneration (literature reviewed by Weiss, '25), while its presence is not required during the ontogenetic development of the limb. 5) The full quota of nerve supply is available from the beginning of limb regeneration, the new fibers arising from the cut ends of the old nerve trunks leading to the limb. This is in contrast to embryonic development, where no comparable numbers of nerve fibers are present when the limb bud first differentiates. Figure 1 illustrates point 5 mentioned above.

Although the regeneration of the urodele limb seems to furnish an excellent object on which to study the factors controlling the quantity and pattern of nerve fibers in a developing organ, there has been only one attempt hitherto to study

the development of nerves within regenerating limbs, namely that of Weiss and Walker ('34). All the earlier work mentioning the nervous system in relation to limb regeneration has been concerned with the question of whether the regeneration of the limb as such was dependent upon influences of the nervous system. The work of Wolff ('02), Hines ('05), Goldfarb ('09, '11), Weiss ('25), and Schotté ('26), were directed to this objective. The investigation of Weiss and Walker ('34), however, specifically attacked the reciprocal problem of defining the role of the regenerating limb in controlling its subsequent innervation. Briefly, they found that the size

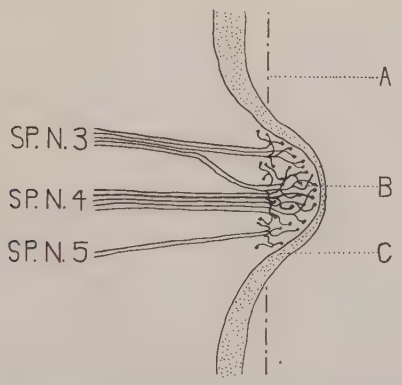

Fig. 1 Diagram to illustrate the invasion of the regenerate by nerve sprouts from the old stump. A, level of limb amputation; B, regenerating nerve sprouts; C, skin.

of the nerve trunks between the centers and base of the regenerated limbs was according to a rough estimate not affected by amputation and subsequent regeneration. However, within the undersized regenerates a reduction had occurred and as an extreme instance of this, cases are mentioned in which the fore limb had failed to regenerate, and where it was subsequently found that although the trunks of the third and fourth spinal nerves of the two sides (control and operated) corresponded in size, the nerves on the operated side stopped at the level of the plexus, giving off only a few thin branches to girdle muscles. Consequently, a certain relation

between the mass of regenerated limb tissues and the amount of nerve regeneration was strongly indicated.

This emphasis upon a correlation between the limb itself and the amount of its innervation demanded a more detailed study, the more so as Weiss and Walker's conclusions were based on estimates only and not on exact quantitative determinations. A detailed account of the relation between the mass of a regenerated limb and the number of nerve fiber branches grown into it seemed therefore promising and desirable. This paper deals with the quantitative relations which are found between the regenerating nerves and the regenerating limb and includes a comparative study of the number of myelinated and non-myelinated nerve fibers. Regenerates were studied at different ages and stages of regeneration: Thus a graded series of successively greater peripheral masses of limb tissue was obtained while the nerve supply at the cut surface ready to invade the regenerate was of constant size in all cases. A preliminary account of the results has been given in Weiss and Litwiller ('37).

MATERIAL AND METHODS

Two species of urodeles were used, the Japanese newt, Triturus pyrrhogaster (Boie) and the Mexican axolotl, Amblystoma mexicanum Cope. The aim in using two species was to throw further light upon our problem, through differences, that might be merely incident to the rate of regeneration, metamorphosis and size of animals concerned. A total of 200 newts and 20 axolotls were used in the experiments. The animals were fed on a diet of beef liver every 3 to 4 days. All were kept individually in either earthenware crocks of 1-gallon capacity or in small finger bowls, in both cases filled with about 1 inch of (chlorinated) city water.

Operations were performed on the right anterior limb, using the left limb as control. The amputations were performed in the following manner: The animals were anaesthetized, Triturus in a mixture of ether and air, Amblystoma in a saturated chloretone solution. The limb was amputated at its base just distal to the proximal head of the humerus.

Because of the retraction of the muscles from the cut, the humerus invariably projected from the wound, which necessitated a second amputation, this time of the protruding end of the humerus.

The operated axolotls were arranged into two groups differing only in size as judged by average lengths. The odd protocol numbers as used in this paper, represent the small axolotls averaging from 75 to 85 mm. in length, while the even protocol numbers represent a group whose average length ranges from 120 to 150 mm., measured from mouth to the tip of the tail. Regeneration was allowed to proceed for varying periods of time; at the end of this the limbs were removed

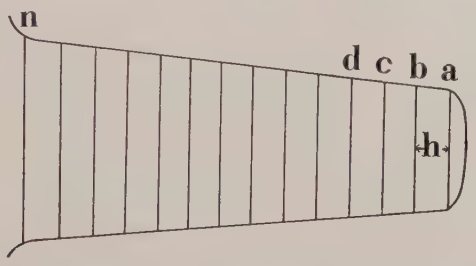

Fig. 2 Diagram to illustrate the computation of peripheral volume of regenerating limb. a, b, c, d . . . . n, represent areas of cross sections of the regenerated limb; h, represents the distance of 200 μ between consecutive levels.

including the shoulder region and fixed. The intervals between amputation and termination of the experiments varied from 25 to 118 days for Triturus, and from 5 to 65 days for Amblystoma. These different time scales in the two species correspond approximately to each other because regeneration takes place more rapidly in the larval axolotl than in the metamorphosed newt. In both species the observed intervals exceed amply the time necessary for the perfection of well-formed and functional limbs. In all cases the opposite limbs were preserved along with the experimental limbs as controls. The regenerates and their control limbs were treated either by Bielschowsky's or Weigert-Pal's method, depending upon whether the total number of nerve fibers were wanted or only myelinated fibers. The limbs were straightened when fixed and sectioned at right angles to their long axes.

Sections were cut at 10 μ; every twentieth section was chosen for study, beginning at the base of the limb, thus allowing a distance of 200 μ between successive sample levels. The method for counting the nerve fibers and determining the area of the cross section has been described in detail by Litwiller ('38). The nerve fibers were counted with the aid of a camera lucida. The area of the cross sections were determined, outlining them by means of a camera lucida upon graph paper (100 units per square inch). Since the same degree of magnification, × 43, was used in all cases, the number of squares covered gives the size of the cross section in arbitrary units.

In order to determine the volume of limb tissue distal to any given level, the following method was used: If a regenerating limb is considered as a solid structure of known outline and if the area of the section at level *a* is known to be of the size of *a,* and that of *b* to be of size *b,* and if *h* is the height of the slice, 200 μ, constant between all sections, the volume of any fraction of the limb would be

$$V_{an} \cong h \cdot \sum_{a}^{n-1} s \qquad (1)$$

where s means sectional area, $\sum_{n}^{a} s = a + b + c + d + \ldots + n$, and *n* is the number of slices contained in that fraction.

To simplify the determinations, the sum of the cross sections were used instead of determining the volume. This can be done since *h* is a constant, common to all regenerated limbs.

Experiments on the quantitative control of nerve fibers in regenerating limbs

a. Determination of the number of myelinated and non-myelinated nerve fibers within regenerated and normal limbs. A quantitative study of the ratio between myelinated and non-myelinated nerve fibers has, to the author's knowledge, never been reported on regenerated or normal urodele limbs. Since the silver impregnations which reveal the total nerve content of a limb, often fail on material in which myelin stains encounter no difficulty, it was necessary to establish the standard proportion of myelinated fibers so that later

the total number could be estimated by extrapolation. Thus an immediate comparison between results obtained with different staining technics was made possible. Bielschowsky's silver method did not prove very successful on the limbs of Amblystoma, whereas the Weigert procedure for myelin was used with considerable success. On the other hand, although it was possible to obtain satisfactory myelin stains of Triturus control limbs, it was found that myelination is absent in the stages of regeneration needed for our studies, as judged by Weigert-Pal stains. These early stages of Triturus regenerates were needed, however, in the quantitative comparison of the number of nerve fibers within limbs at various stages of regeneration. In consequence, myelin stains were used mainly upon the regenerated limbs of Amblystoma, while Bielschowsky's silver methods had to be applied to the limbs of Triturus.

There are a few successful cases, however, in which in the same specimen a successful stain was obtained in both forelimbs, one being treated with silver and the other with the myelin stain. These cases permitted a reliable determination of the proportion of myelinated to the total number of nerve fibers in urodele limbs.

Table 1 shows the nerve counts at the base of the limbs (regenerated) of Amblystoma, including two cases of small animals and one of the larger group.

TABLE 1

Amblystoma. Comparison of counts of myelinated fibers with total number of fibers

	NUMBER OF MYELINATED FIBERS (LEFT LIMB)	TOTAL NUMBER OF FIBERS (RIGHT LIMB)	PER CENT OF MYELINATED FIBERS
Small animals (75–80 mm.)			
AM 19 50 day regenerate body length 75 mm.	1001	1217	82.2
AM 17 45 day regenerate body length 80 mm.	884	1126	78.4
Large animal (133 mm.)			
AM 18 60 day regenerate body length 133 mm.	1282	1586	80.8

It will be seen from this table that about 80% or four-fifths of the total number of fibers entering the regenerated limb are myelinated fibers.

Data are also available for two control Triturus limbs from two different animals, one limb being treated for total nerve fiber number and the other for myelinated fibers only.

TABLE 2

Triturus. Comparison of ratios, $\frac{\text{nerve number}}{\text{area of cross section}}$, of limbs stained for myelinated fibers with those stained for the total number of nerve fibers

TN 1 LENGTH FROM JAW TO ANUS, 56 MM. STAINED FOR MYELINATED FIBERS (WEIGERT-PAL METHOD)				TN 14 LENGTH FROM JAW TO ANUS, 39 MM. STAINED FOR TOTAL NUMBER OF FIBERS (BIELSCHOWSKY METHOD)			PERCENTAGE OF MYELINATED FIBERS $100 \cdot \frac{n_m \cdot a'}{n_t \cdot a}$
Level of count	Number of myelinated nerve fibers n_m	Area of limb cross section a	Ratio of nerve number to area $\frac{n_m}{a}$	Total number of nerve fibers n_t	Area of limb cross section a'	Ratio of nerve number to area $\frac{n_t}{a'}$	
Base of limb	1690	2171	0.780	1324	1446	0.915	85.2
Elbow	802	1480	0.541	1101	1611	0.684	79.1
Upper wrist	347	1020	0.340	651	1435	0.453	75.0

If differences in size of the limb are accounted for, and this can be done roughly by dividing the number of nerve fibers at any given level by the area of the cross section giving the ratio n/a, the two limbs can be compared directly. It is assumed that the number of nerve fibers in a given section is roughly proportional to the area. Evidence of this will be presented below.

A comparison made at the base of the limb reveals a value of 0.915 for the total number of fibers and 0.780 for the myelinated number, the difference being 14.8%. At this level 85.2%of the nerve fibers are myelinated fibers. Similarly, at the elbow there is a difference of 20.9%, or 79.1% of the fibers are myelinated. At the wrist region, a difference of 25.0% is found or again, 75.0% of the fibers are myelinated.

It follows then that the percentage of myelinated fibers ranges between 78 to 83, irrespective of age, size and age of

regeneration in Amblystoma. The results obtained with adult metamorphosed Triturus limbs are essentially similar to Amblystoma.

b. Determination of the number and density of nerve fibers in regenerated limbs. In an effort to determine the relationship between the number of nerve fibers found at any given level of a regenerating limb and the size of the non-nervous area as an expression of the mass of tissue, every twentieth section from the base of the limb to the wrist was studied.

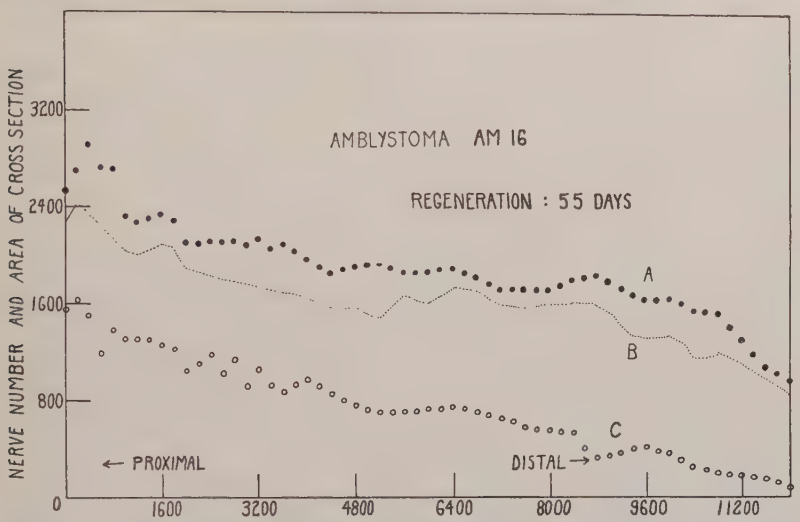

Fig. 3 Large Amblystoma, 142 mm. Graph shows area of cross sections and the number of myelinated nerve fibers within them. Abscissa, limb axis; A, area of limb cross section; B, area of cross section of limb, minus cartilage; C, number of myelinated nerve fibers.

Table 3 [3] illustrates an example; similar tables were made for each case investigated. The data obtained have been plotted in various ways in graph form in an effort to determine correlations controlling the numbers and grouping of the entering nerve fibers within the limb.

Figure 3 illustrates clearly that as more peripheral sections are plotted a steady and more or less parallel decline in both nerve number and cross sectional areas of the limb occurs.

[3] Tables 3, 4 and 5 have been placed on file at The Wistar Institute and may be referred to if desired.

The strongest evidence of the existence of a definite relation between the number of fibers at any level of the limb and the size of that level, came from the fact that when the number of nerve fibers of a regenerated limb was divided by the area of its respective cross section, a value—the relative density of nerve fibers (n/a)—was obtained which, when compared to the corresponding value at every twentieth section throughout the regenerate, remained within the same order of magnitude, in spite of considerable fluctuations from section to section. In table 4 these n/a ratios at every twentieth section are listed from eight cases of Amblystoma regenerates, varying as to age of regeneration as well as to size of animal.

A close study of these ratios reveals the following facts: 1) The values for the relative nerve density decreases steadily but slowly from base to more distal levels of the limb. This general trend in the reduction of n/a values is also supported by the results of similar determinations for the regenerated limbs of Triturus. 2) In table 4 the ratios, n/a of any one regenerate agree well with ratios of other regenerates of different ages of regeneration, provided we remain within a given size group. 3) The averages of the n/a ratios of the regenerates of the large group of Amblystoma are less than the ratios found for the smaller group.

The first point noted in connection with these ratios, namely that they tend to decrease uniformly from the base to the wrist, indicates that the relative density of fibers, if calculated for the total area of tissue, is greater at proximal levels than at more distal levels, or that there are fewer fibers per unit of total area distally than proximally. Part of this decline may be due to the inclusion of cartilage, a tissue which assumes an increasingly greater fraction of the total area in more distal levels, particularly the wrist. In calculations made for the soft tissues alone, the relative density of nerve fibers shows less decline than is suggested by the above data.

Notwithstanding this correction, there still is a significant proximo-distal decline in the nerve fiber density, and it was this fact that furnished a lead toward a more appropriate

correlation than that with area. The one factor that obviously decreases in a proximo-distal direction as steadily as the nerve density is the volume of limb tissue lying distal to the successive levels. To obtain the exact values we proceeded according to the method outlined in figure 2. After calculating the volume of limb lying distal to any given level, graphs were made, plotting these values on the abscissa against the number of nerve fibers on the ordinate.

All of the regenerated limbs of both Amblystoma and Triturus as well as sample control limbs of Triturus were studied, and the data treated in the above manner. Samples of the type of graphs obtained are presented in figures 4 and 5.

It will be observed (fig. 4) that the graphs furnish a series of points which very nearly determine a straight line. This linear relationship was equally striking in all studied cases. In this particular series (fig. 4) five different regenerate limbs, each representing a different age of regeneration are shown. Since all five animals belonged to the same group of Amblystoma (130 to 150 mm. body length), the only essential variable among them is the difference in age of regeneration.

Several facts stand out from a study of the five graphs in figure 4: 1) The course of the scattered points can be fitted in each case, with fair approximation, by a straight line, indicating a simple direct proportion between the number of nerve fibers at any level and the volume of the limb distal to that particular level. 2) With successively increasing age of regeneration (from top to bottom in fig. 4), the slope of the line decreases. This is due to the fact that the number of original nerve fibers present at the base of the regenerating limbs remains practically the same, amounting to about 1200 fibers on an average, whereas the volume of the limbs increased with increasing age of regeneration. Thus the drop of the line from a value near 1200 for the most proximal end to almost zero at the distal end, takes place over a longer range in the older more voluminous regenerates, resulting in a slope of lesser decline. This, at the same time, means that the ratio

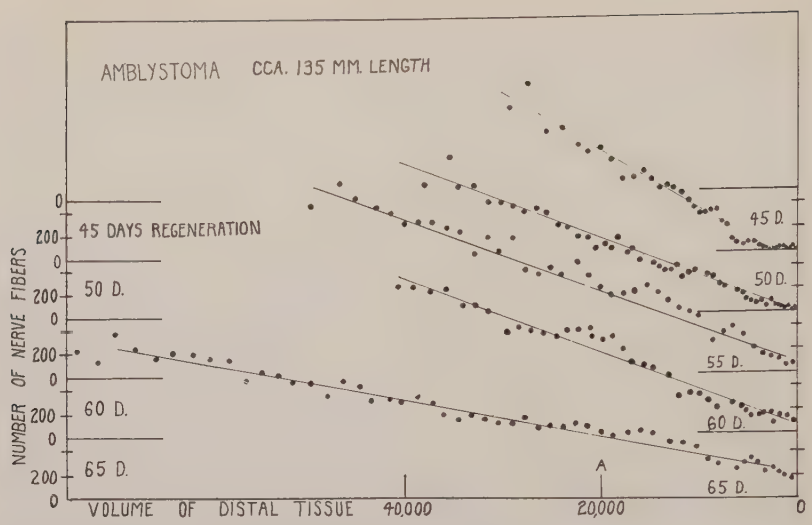

Fig. 4 Graphs of regenerated limbs of large Amblystoma, animals 130 to 140 mm. in length. Ordinate: The number of myelinated nerve fibers at corresponding level. Abscissa: Volume of limb tissue peripheral to level.

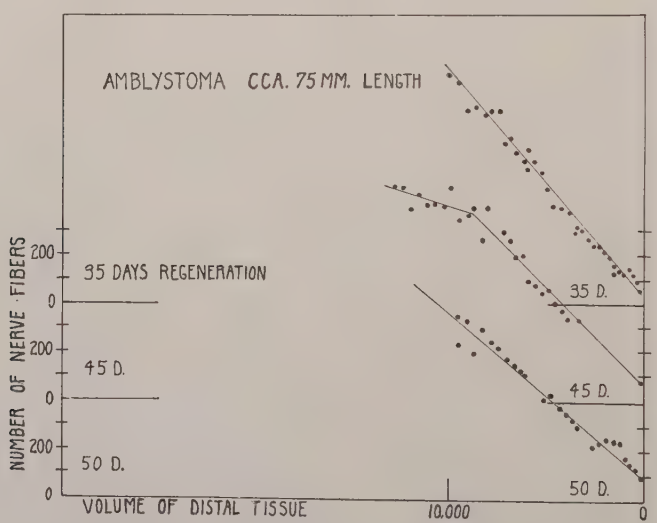

Fig. 5 Similar graphs as in figure 4, but of small Amblystoma, 75 to 80 mm. Scale of both ordinate and abscissa twice that of figure 4.

of nerve fibers per unit of distal volume decreases with increasing ages of regeneration. Reduced to the unit of innervated volume (ratio of nerve number to distal volume, n/v_D) there are relatively more nerve fiber branches present in the younger regenerates than in the older ones. For instance, the level leading to a distal mass of tissue labeled (A)

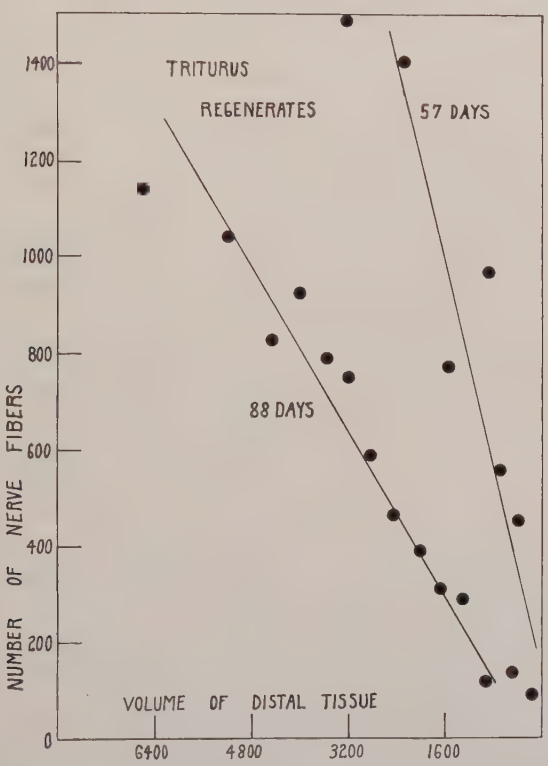

Fig. 6 Graph of Triturus regenerates of 57 and 88 days. Abscissa: Volume of limb tissue distal to level. Ordinate: Number of nerve fibers at corresponding level.

in figure 4 or 20,000 units is passed by about 880, 750, 500 nerve fibers in regenerates of 45, 55 and 65 days of age, respectively.

This consistent difference in the slope of the curves pertaining to different ages of regeneration is equally characteristic of regenerating limbs of the same age of regeneration but produced by animals differing in age and body size.

Thus, if the first and second curves of figure 4, representing regenerates of 45 and 50 days, respectively, in large animals, are compared with the second and third curves of figure 5, representing stages of equal ages of regeneration but in small animals, the considerably greater decline of the curves of the latter can be seen.

Combining the results of the two sets, we may conclude, then, that the amount of the nerve fiber branches per unit of distal

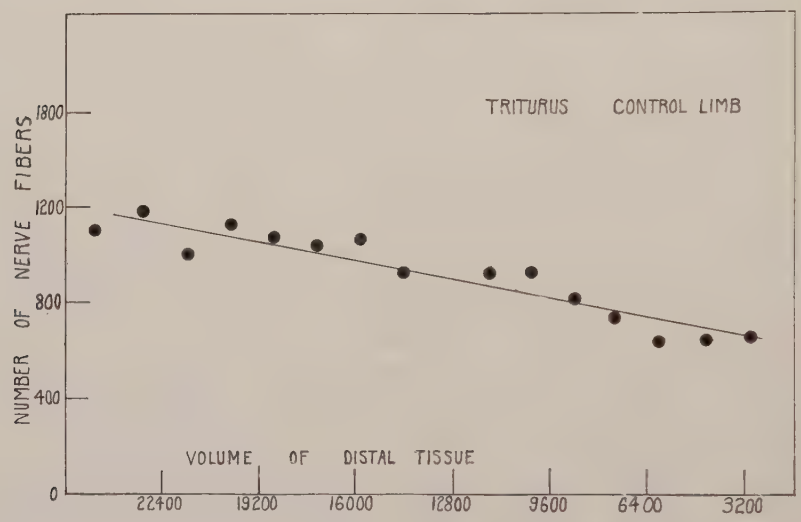

Fig. 7 Graph of normal Triturus limb; scale twice that used in figure 6.

mass to be innervated (n/v_D) is approximately constant throughout a given regenerate, but decreases steadily both with age of the animal and with the age of regeneration.

The graphs of Triturus (figs. 6 and 7) corroborate essentially the finding obtained in Amblystoma. For example it was found that as the age of regeneration increases (57 to 88 days, fig. 6) the number of nerve fiber branches per unit of distal volume decreases, as indicated by a decrease in the slope of the respective curves. The corresponding curve of a normal Triturus limb can be seen in figure 7. In figure 7 the scale of both ordinates and abscissa is twice that of figure 6.

DISCUSSION

The number of myelinated nerve fibers entering a regenerating limb proved to be rather constant within the same size group of Amblystoma. In the small group this average centers around 1000 medullated nerve fibers, while in the larger group it averages about 1200. As for Triturus the total number of myelinated and unmyelinated nerve fibers entering the fore limb is listed in table 6.

TABLE 6

NUMBER	NUMBER OF ENTERING FIBERS	LIMB IN REGENERATION FOR
Triturus TN 14	1324	Normal limb
Triturus TB 10	1138	106 days
Triturus TB 14	1216	96 days
Triturus TB 16	1020	91 days
Triturus TB 1	1049	75 days
Triturus TB 30	1117	72 days
Triturus TB 34	1418	64 days
Triturus TB 37	1284	57 days
Triturus TB 38	1482	57 days
Triturus TB 39	1041	51 days
Triturus TB 40	1217	51 days
Triturus		
Average	1210	

In general, the number entering the operated and control limbs are similar. Moreover, the number of nerve branches which are admitted to the growing limb is independent of the age of regeneration. For example, at 106 days of regeneration (TB 10) 1138 nerve fiber branches entered the base of the regenerate. The corresponding number in a regenerate of only 51 days (TB 40) was found to be 1217. In some cases, for example TB 34 and 38, the number of fibers counted is greater in early regenerates, but this is probably due to the extensive branching at the base of the limb and the great difficulty of determining the boundaries of the future definitive limb in the early regenerates.

At the base of the regenerate the full nerve number of a normal limb was counted, this number, as we have seen above drops gradually as we proceed distally through the regenerate.

The main result of this paper is to have demonstrated that the drop occurs not casually but bears a very strict relationship to the distal decrease in mass of tissue to be innervated. It has been shown that at any level of a regenerated limb, the ratio between number of nerve fibers passing through it and amount of tissue distal to it is constant for a given size class and a given age of regeneration. This is expressed by the linear course of the graphs (figs. 4, 5, 6, 7) and can be written in the form of an equation:

$$n/v_D = k$$

where n is the number of nerve fiber branches and v_D is the volume distal to the section in question. This ratio k consistently decreases with increasing age of regeneration. This

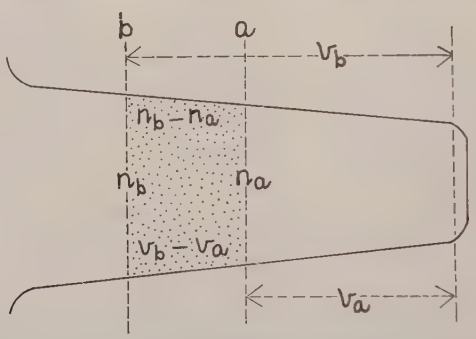

Fig. 8 Diagram to illustrate the relation between terminating nerve fibers and the volume of the slice in which they terminate.

suggests that the production of peripheral nerve branches probably proceeds at a lower rate than the growth of the non-nervous periphery. It has also been shown that the constant k is greater in the younger than in the older specimens of Amblystoma, other conditions equal.

Next the question arises as to the concrete meaning of this simple formal relationship: It must be remembered that every fiber count at a more proximal level includes the full number of branches counted at any of the more distal levels (provided there is no appreciable branching of the fibers

during their further distal course). Therefore, the difference between fiber counts taken at any two levels equals the number of nerve fibers that have come to terminate in the mass of tissue bounded by the two levels. Figure 8 and calculations illustrate the situation:

n_a = number of nerve fibers at level a
n_b = number of nerve fibers at level b
$n_b - n_a$ = number of nerve fibers which have terminated between levels b and a
v_a = volume distal to level a
v_b = volume distal to level b
$v_b - v_a$ = volume between levels b and a

We have demonstrated in the text that:

1) $$n/v = k$$

Therefore, for level a:

2) $$\frac{n_a}{v_a} = k, \text{ or } n_a = k \cdot v_a$$

Likewise for level b:

3) $$n_b = k \cdot v_b;$$

subtracting (2) from (3):

4) $$n_b - n_a = (k \cdot v_b) - (k \cdot v_b) = k\,(v_b - v_a)$$

or,

5) $$\frac{n_b - n_a}{v_b - v_a} = k;$$

and for any arbitrary levels

6) $$\frac{n_n - n_m}{v_n - v_m} = k;$$

where $(v_n - v_m)$ represents the volume of the slice between the levels and $n_n - n_m$ represents the number of nerve terminals therein. We thus arrive at the conclusion that the number of nerve fibers terminating within any slice of the limb selected at random, is in direct proportion to the volume of the slice. This means that the number of nerve endings per unit of tissue mass is a constant k within a given regenerate. To illustrate, using data from regenerate AM 14 (table 3) the number of nerve fibers at level 6 is 1075 and the number at level 9 is 910. Their respective volumes peripheral to the

levels are 48,496 and 42,498 units, respectively. These figures expressed in the formula above would be:

1) $$k_{nm} = \frac{1075 - 910}{48{,}496 - 42{,}498} = 0.0275$$

or again at levels 10 and 20,

$$= \frac{884 - 563}{40{,}789 - 26{,}845} = 0.0230$$

To calculate in terms of actual limb volumes rather than arbitrary units we must proceed as follows: Using the formula developed earlier,

$$v_{ad} = h\left(\frac{a}{2} + b + c + \frac{d}{2}\right)$$

the volume between levels 6 and 9 of AM 14 would be:

$$v_{6,9} = h\left(\frac{1709}{2} + 2003 + 2040 + \frac{1955}{2}\right)$$

$$v_{6,9} = h\ (5872)$$

Since each unit equals 1/100 square inch (see p. 382) or 6.45 mm.², this number of 5872 converted into mm.² would be $5872 \cdot 6.45 = 37{,}874.4$ mm.². The magnification value 1849 (43^2) divided into 37,874.4 mm.² is 20.49 mm.². Since h equals 200 μ or 0.2 mm., the actual volume of the slice $v_{6,9} = 4.098$ cu.mm. It will be recalled that the number of nerve fibers which have terminated between levels 6 and 9 was 1075 — 910 or 165, consequently:

$$k_{6,9} = \frac{165 \text{ fibers}}{4.098 \text{ cu.mm.}} = 40.28 \text{ fibers/cu.mm.}$$

The number of nerve fiber branches ending in 1 cu.mm. is thus calculated to be about forty nerve fibers. The reciprocal value 1/k, that is the average mass per single nerve fiber termination is found to be 0.0215 cu.mm. This clearly shows that the mass of non-nervous tissue exerts a definite limiting influence upon the amount of nerve fiber terminals formed within it.

It will be recalled that the k values expressed by the slope of the curves in figures 4, 5, 6 and 7 were smaller for the older regenerates. This definite decrease indicates that maximum saturation of the regenerating mass with nerve fibers

must have occurred very early in regeneration; that is to say, the relative distribution of 'nerve terminals' throughout the mass of the regenerate is established while the regenerate is still in a relatively primitive condition and the subsequent growth of the regenerate does not appreciably alter the original distribution. Thus, our data not only demonstrate the fact of a control of nerve admission on the part of the limb tissues to be innervated but also suggest that this control does not consist in a secondary elimination of excessive nerve fiber branches under the guidance of functional activity. Rather, this control is primarily exerted by the tissues in early prefunctional stages of differentiation. What part is played by the various tissues as a whole or by its various constituents, such as muscles, skin, cartilage, etc., cannot be decided from our data. In an earlier set of experiments, (Litwiller, '38) regeneration of nerve stumps, after transection, into adult limbs of Triturus modified in various ways, was found to be controlled by the limbs. Whereas in this earlier set, the degenerated peripheral fragments may have exerted part of the control, the results obtained in this paper show that control of innervation is exerted by the regenerating limb in spite of the absence of degenerated nerve sheaths.

SUMMARY

1. Limbs of the Japanese newt, Triturus pyrrhogaster (Boie) and the axolotl, Amblystoma tigrinum (Green) were amputated at their bases and allowed to regenerate for varying periods of time. For the newt, this period was from 30 to 118 days; for the axolotl, from 6 to 65 days. The regenerated limbs were treated with either Bielschowsky's silver method, impregnating all the nerve fibers present, or with a modification of the Weigert-Pal method, staining only the myelinated fibers.

2. Counts of the number of nerve fibers were made throughout the length of the limb at regular intervals of 200 μ and in addition the size of the cross sectional area of the limb at that level was determined.

3. The number of myelinated fibers of Triturus and Amblystoma stained by Weigert-Pal method, averages about 20% less than the total number of fibers stained by Bielschowsky's silver reduction technique. This holds for normal limbs of Triturus as well as for regenerated limbs of Amblystoma. The total number of myelinated fiber branches entering a regenerating limb averages, in the newt, 1210 and in the axolotl about 1000 for the small samples and 1200 for the large ones. These values are independent of the condition and age of regeneration in the free limb.

4. From this basal level the number of nerve fibers drops steadily as one proceeds distally through the limb. This decline, caused by termination of nerve fiber branches at various levels, was found to have not random character but to follow a rather strict rule:

5. A definite linear relation was found to exist between the number of nerve fibers at any given level and the volume of the limb peripheral to that particular level. Expressed in other terms this relation means, that the number of nerve fibers terminating within any arbitrary slice of a regenerate is in direct proportion to the mass of that slice, with a proportionality constant k.

6. The proportionality constant k equals the number of nerve fiber branches ending in a unit of tissue mass. This constant has been found to decline with increasing age of regeneration, as well as with increasing ages of the animals.

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